Amendments to the Specification

Please replace the paragraph beginning at page 3, line 31 with the following amended paragraph:

The present invention aims at providing means for efficient diagnosis of infections with Chlamydia pneumoniae as well as the development of effective vaccines against infection with this microorganism. The invention thus relates to species specific diagnostic tests for infection in a mammal, such as a human, with Chlamydia pneumoniae, said tests being based on the detection of antibodies against surface exposed membrane proteins of a size of approximately 89-101 kDa and of 56-57 kDa, preferably of about 89.6-100.3 kDa and about 56.1 kDa (the range in size of the deduced amino acid sequences was from 100.3 to 89.6 except for Omp13 SEQ ID NO:20 with the size of 56.1 kDa), or the detection of nucleic acid fragments encoding such proteins or variants or subsequences thereof. The invention further relates to the amino acid sequences of proteins according to the invention, to variants and subsequences thereof, and to nucleic acid fragments encoding these proteins or variants or subsequences thereof. The present invention further relates to antibodies against proteins according to the invention. The invention also relates to the use of nucleic acid fragments and proteins according to the invention in diagnosis of Chlamydia pneumoniae and vaccines against Chlamydia pneumoniae.

Please replace the paragraph beginning at page 6, line 5 with the following amended paragraph:

Mice infected with C. pneumoniae generate antibodies to the proteins identified by the inventors and named Omp4-15 (SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24), but do not recognize the SDS treated heat denatured antigens normally used for SDS-PAGE and immunoblotting. However, a strong reaction was seen if the antigen was not heat denatured. It is therefore highly likely that if a similar reaction is seen in connection with human infections the antigens of the present invention will

be of invaluable use in sero-diagnostic tests and may very likely be used as a vaccine for the prevention of infections.

Please replace the paragraph beginning at page 6, line 16 with the following amended paragraph:

By generating antibodies against COMC from C. pneumoniae a polyclonal antibody (PAB 150) was obtained which reacted with all the proteins. This antibody was used to identify the genes encoding the 89.6-101.3 kDa and 56.1 kDa proteins in an expression library of C. pneumoniae DNA. A problem in connection with the present invention was that a family comprising a number of similar genes were found in C. pneumoniae. Therefore, a large number of different clones were required to identify clusters of fragments. Only because the rabbit antibody generated by the use of SDS-denatured antigens contained antibodies to a high number of different epitopes positioned on different members of the protein family did the inventors succeed in cloning and sequencing four of the genes. One gene was fully sequenced, a second was sequenced except for the distal part and shorter fragments of two additional genes were obtained by this procedure. To obtain the DNA sequence of the additional genes and to search for more members of the gene family long range PCR with primers derived from the sequenced genes, and primers from the genes already published in the database were used. This approach gave rise to the detection of additional eight genes belonging to this family. The genes were situated in two gene clusters: Omp12,11,10,5,4,13 and 14 (SEQ ID NOs:17, 15, 13, 3, 1, 19, 21) in one cluster and Omp6,7,8,9 and 15 (SEQ ID Nos:5, 7, 9, 11, 23) in the second. Full sequence was obtained from Omp4,5,6,7,8,9,10,11 and 13 (SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 19), and partial sequence of Omp12,14 (SEQ ID NOS:17, 21). Omp13 (SEQ ID NO:19) was a truncated gene of 1545 nucleotides. The rest of the full length genes were from 2526 (Omp7) (SEQ ID NO:7) to 2838 (Omp15) (SEQ ID NO:23) nucleotides. The deduced amino acid sequences revealed putative polypeptides of 89.6 to

100.3 kDa, except for Omp13 (SEQ ID NO:20) of 56.1 kDa. Alignment of the deduced amino acid sequences showed a maximum identity of 49% (Omp5/Omp9) (SEQ ID NO:4)/(SEQ ID NO:12) when all the sequences were compared. Except for Omp13 (SEQ ID NO:20), the lowest homology was to Omp7 (SEQ ID NO:8) with no more than 34% identity to any of the other amino acid sequences. The scores for Omp13 (SEQ ID NO:20) was from 29-32% to all the other sequences.

Please replace the paragraph beginning at page 7, line 24 with the following amended paragraph:

The estimated size of the Omp proteins ~~of the~~ of the present invention are listed in the following. Omp 4 (SEQ ID NO:2) has a size of 98.9 kDa, Omp5 (SEQ ID NO:4) has an estimated size of 97.2 kDa, Omp6 (SEQ ID NO:6) has an estimated size of 100.3 kDa, Omp7 (SEQ ID NO:8) has an estimated size of 89.7 kDa, Omp8 (SEQ ID NO:10) has an estimated size of 90.0 kDa, Omp9 (SEQ ID NO:12) has an estimated size of 96.7 kDa, Omp10 (SEQ ID NO:14) has an estimated size of 98.4 kDa, Omp11 (SEQ ID NO:16) has an estimated size of 97.6 kDa, Omp13 (SEQ ID NO:20) has an estimated size of 56.1 kDa, Omp 12 and 14 (SEQ ID NO:18) and (SEQ ID NO:22) being partial.

Please replace the paragraph beginning at page 8, line 21 with the following amended paragraph:

The full length Omp4 (SEQ ID NO:1) was cloned into an expression vector system that allowed expression of the Omp4 polypeptide (SEQ ID NO:2). This polypeptide was used as antigen for immunization of a rabbit. Since the protein was purified under denaturing condition the antibody did not react with the native surface of C. pneumoniae, but it reacted with a 98 kDa protein in immunoblotting where purified C. pneumoniae EB was used as antigen. Furthermore, the antibody reacted in paraffin embedded sections of lung tissue from experimentally infected mice.

Please replace the paragraph beginning at page 14, line 18 with the following amended paragraph:

By the term "similar biological function" is meant that the protein shows characteristics similar with the proteins derivable from the membrane proteins of *Chlamydia pneumoniae*. such proteins comprise repeated motifs of GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2) (at least 2, preferable at least 3 repeats) and/or conserved positions of tryptophan, (w).

Please replace the paragraph beginning at page 14, line 24 with the following amended paragraph:

Comparison of the DNA sequences from genes encoding Omp4-15 (SEQ ID NOs:1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23) shows that the overall similarity between the individual genes ranges between 43-55%. Comparison of the amino acid sequences of Omp4-15 (SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24) shows 34-49% identity and 53-64% similarity. The homology is generally scattered along the entire length of the deduced amino acids. However, as seen from figure 8 A - J there are some regions in which the homology is more pronounced. This is seen in the repeated sequence where the sequence GGAI is repeated 4-7 times in the genes. It is interesting that the DNA homology is not conserved for the sequences encoding the four amino acids GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2). This may indicate a functional role of this part of the protein and indicates that the repeated structure did not occur by a duplication of the gene. In addition to the four amino acid repeats GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2) a region from amino acid 400 to 490 has a higher degree of homology than the rest of the protein, with the conserved sequence FYDPI (Phe-Tyr-Asp-Pro-Ile, amino acids 374-378 of SEQ ID NO:2) occurring in all sequences. As further indication of similarity in function the amino acid tryptophan (W) is perfectly conserved at 4-6 localizations in the C-terminal part of the protein.

Please replace the paragraph beginning at page 15, line 24 with the following amended paragraph:

Preferred embodiments of the present invention relate to polypeptides which comprise subsequences of the proteins of the invention, said subsequences comprising the sequence GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2). Further preferred embodiments of the present invention relate to polypeptides which comprise subsequences of the proteins of the invention, said subsequences comprising the sequence FSGE Phe-Ser-Gly-Glu, amino acids 407-410 of SEQ ID NO:2).

Please replace the paragraph beginning at page 22, line 21 with the following amended paragraph:

Figure 6. The figure shows ~~sequence~~ sequencing strategy for Omp4 (SEQ ID NO:1) and Omp5 (SEQ ID NO:3). Arrows indicates primers used for sequencing.

Please replace the paragraph beginning at page 22, line 24 with the following amended paragraph:

Figure 7. *C. pneumoniae* omp genes. The genes are arranged in two clusters. In cluster 1 Omp12, 11, 10, 5, 4, 13, and 14 (SEQ ID NOs:17, 15, 13, 3, 1, 19, 21) are found. In cluster 2 are found Omp6, 7, 8, 9, and 15 (SEQ ID NOs:5, 7, 9, 11, 23).

Please replace the paragraph beginning at page 22, line 26 with the following amended paragraph:

Figure 8 A - J. The figure shows alignment of *C. pneumoniae* Omp4-15 (SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24), using the program pileup in the GCG package.

Please replace the paragraph beginning at page 22, line 28 with the following amended paragraph:

Figure 9. The figure shows immunofluorescence of *C. pneumoniae* infected HeLa, 72hrs. after infection, reacted with mouse monospecific anti-serum against pEX3-36 fusion protein.

pEX3-36 is a part of the Omp5 gene (SEQ ID NO:3).

Please replace the paragraph beginning at page 28, line 6 with the following amended paragraph:

The DNA sequence encoding the Omp4-15 proteins (SEQ ID NOs:2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24) with a size of 89.6-100.3 kDa (and for Omp13 (SEQ ID NO:20): 56.1 kDa). The Omp4 (SEQ ID NO:1) and Omp5 (SEQ ID NO:3) DNAs were transcribed in opposite directions. Downstream of the coding sequence of the Omp4 gene (SEQ ID NO:1) a possible termination structure was located. The 3'end of the Omp5 gene (SEQ ID NO:3) was not cloned due to the presence of the BamHI restriction enzyme site positioned within the gene. The amino acid (translated DNA) sequences of Omp4 (SEQ ID NO:2) and Omp5 (SEQ ID NO:4) ~~was~~ were compared by use of the gap programme in the GCG package (Wisconsin package, version 8.1-UNIX, August 1995, sequence analysis software package). The two ~~genes~~ translations had an amino acid identity of 41% (similarity 61%), and a possible cleavage site for signal peptidase 1 was present at amino acid 17 in Omp4 (SEQ ID NO:2) and amino acid 25 in Omp5 (SEQ ID NO:4). When the amino acid sequence encoded by two other pEX clones were compared to the sequence of Omp4 (SEQ ID NO:2) and Omp5 (SEQ ID NO:4) they also had amino acid homology to the genes. It is seen that the two clones have homology to the same area in the Omp4 (SEQ ID NO:2) and Omp5 (SEQ ID NO:4) proteins. Consequently, the pEX clones must have originated from two additional genes. Therefore these genes were named Omp6 (SEQ ID NO:6) and Omp7 (SEQ ID NO:8). Similar analyses were performed with the other genes. In contrast to what was seen for Omp4 (SEQ ID NO:2) and 5 (SEQ ID NO:4) none of the other putative omp proteins had a cleavage site for signal peptides.

Please replace the paragraph beginning at page 28, line 29 with the following amended paragraph:

Polyclonal monospecific antibodies against pEX fusion

proteins and full length recombination + Omp4 (SEQ ID NO:2)

Please replace the paragraph beginning at page 28, line 31 with the following amended paragraph:

To investigate the topology of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8), representative pEX clones, were selected from each gene. The fusion proteins of β-galactosidase/omp were induced, and the proteins were partially purified as inclusion bodies. Balb/c mice were immunized three times intramuscular with the antigens at an interval of one week, and after six weeks the serum was obtained from the mice. HeLa cells were infected with the C. pneumoniae. 72 hours after the infection the mono-layers were fixed with 3.7% formaldehyde. This treatment makes the outer membrane of the Chlamydia impermeable for antibodies due to the extensive cross-linking of the outer membrane proteins by the formaldehyde. The HeLa cells were permeabilized with 0.2% Triton X100, the monolayers were washed in PBS, then incubated with 20% (v/v) FCS to inactivate free ~~radicals of the formaldehyde. The mice sera were diluted 1:100~~ PBS with 20% (v/v) FCS and incubated with the monolayers for half an hour. The monolayers were washed in PBS and secondary FITCH conjugated rabbit anti mouse serum was added for half an hour, and the monolayers were washed and mounted. Several of the antibodies reacted strongly with the EBs in the inclusions (Figure 9). In spite of the formaldehyde fixation it could not be excluded that the surface of the EB was changed by the treatments, so that the antibodies could get access to the Omp4-7 (SEQ ID NOs:2, 4, 6, 8). Therefore, the reaction was confirmed by immuno-electron microscopy with the antibody raised against clone pEX3-36. Purified EB of C. pneumoniae were absorbed to carbon coated nickel grids. After the absorption the grids were washed with PBS and blocked in 0.5% Ovalbumin dissolved in PBS. The antibodies were diluted 1:100 in the same buffer and incubated for 30 minutes. The grids were washed in PBS. Rabbit anti mouse Ig conjugated with 10nm colloidal gold diluted in PBS

containing 1% gelatin was added to the grids for half an hour. The grids were washed in 3 x PBS with 1% gelatin and 3 times in PBS, the grids were contrastained with 0.7% phospho tungstic acid. The grids were analysed in a Jeol 1010 electron microscope at 40 kV. It was seen that the gold particles were covering the surface of the purified EB. Because the C. pneumoniae EBs were not exposed to any detergent or fixation under either the purification or the reaction with antibodies, these results show that the cloned proteins have surface exposed epitopes.

Please replace the paragraph beginning at page 30, line 3 with the following amended paragraph:

Polyclonal monospecific antibodies against Omp4 (SEQ ID NO:2)

The Omp4 gene (SEQ ID NO:1) was amplified by PCR with primers that contained LIC-sites, and the PCR product was cloned into the pET-30 LIC vector (Novagen). The histidine tagged fusion protein was expressed by induction of the synthesis by ~~IPTG and purified over a nickel column.~~ The purified Omp4 (SEQ ID NO:2) protein was used for immunization of a rabbit (six times, 8 µg each time).

Please replace the paragraph beginning at page 30, line 30 with the following amended paragraph:

The insert of pEX1-1 clone was amplified by PCR using primers containing LIC sites. The PCR product could therefore be inserted in the pET-32 LIC vector (Novagen, UK cat No. 69076-1). Thereby the insert sequence of the pEX1-1 clone was expressed in the new vector as a fusion protein, the part of the fusion protein encoded by the pET-32 LIC vector had 6 histidine residues in a row. The expression of the fusion protein was induced in this vector, and the fusion protein could be purified under denaturing condition on a Ni2+ column due to the high affinity of the histidine residues to divalent cations. The purified protein was used for immunization of a New Zealand white rabbit.

After 6 times intramuscular and 2 times intravenous immunization the serum was obtained from the rabbit. Purified C. pneumoniae EB was dissolved in SDS-sample buffer. Half of the sample was heated to 100°C in the sample buffer, whereas the other half of the sample was not heated. The samples were separated by SDS-PAGE, and the proteins were transferred to nitrocellulose, the serum was reacted with the strips. With the samples heated to 100°C the serum recognized a high molecular weight band of approximately 98 kDa. This is in agreement with the predicted size of Omp5 (SEQ ID NO:4), of which the pEX1-1 clone is a part, however, when the antibody was reacted to the strip with unheated EB, the pattern was different. Now a band was seen with a size of 75 kDa, in addition weaker bands were observed above the band (Figure 10). These data demonstrate that Omp5 (SEQ ID NO:4) needs boiling in SDS-sample buffer to be fully denatured and migrate with a size as predicted from the gene product. When the samples were not boiled, the protein was not fully denatured and less SDS binds to the protein and it has a more globular structure that will migrate faster in the acrylamide gel. The band pattern looked identical to what was obtained with a monoclonal antibody (MAb 26.1)(lane 6), we earlier have described (Christiansen et al., 1994), reacting with the surface of C. pneumoniae EB, but the antibody do not react with the fully SDS denatured C. pneumoniae EB in immunoblotting.

Please replace the paragraph beginning at page 32, line 2 with the following amended paragraph:

Due to the realization of the altered migration of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8) without boiling, we chose to analyse antibodies against C. pneumoniae EBs after an experimental infection of mice. To obtain antibodies from an infection caused by C. pneumoniae, C57 black mice were inoculated intranasally with 10^7 CFI of C. pneumoniae under a light ether anaesthesia. After 14 days of infection the serum samples were obtained and the lungs were analysed for pathological changes.

In two of the mice a severe pneumonia was observed in the lung sections, and in the third mouse only minor changes were observed. The serum from the mice was diluted 1:100 and reacted with purified EBs dissolved in sample buffer with and without boiling. In the preparations that had been heated to 100°C the sera from two of the mice reacted strongly with bands of 60/62 kDa and weaker bands of 55 kDa, but no reaction was observed with proteins of the size of Omp4-7 (SEQ ID NOs:2, 4, 6, 8) (Figure 11). However, when the sera were reacted with the preparation that had not been heated they all had a strong reaction with a broad band of an approximate size of 75 kDa. This is in agreement with the size of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8) in the unheated preparation. Therefore, it could be concluded that the epitopes of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8) recognized by the antibodies after a C. pneumoniae infection were discontinuous epitopes because the full denaturation of the antigen completely destroyed the epitopes. The 75 kDa protein observed in unheated samples is not Omp2 (Shown in immunoblotting with an Omp2 specific antibody).

Please replace the paragraph beginning at page 32, line 30 with the following amended paragraph:

Comparison of Omp4-7 (SEQ ID NOs:2, 4, 6, 8) of C. pneumoniae with putative outer membrane proteins (POMP) of C. psittaci

Longbottom et al. (1996) have published partial sequence from 98 to 90 kDa proteins from C. psittaci. They have entered the full sequence of 5 genes in this family in the EMBL database. They have named the genes "putative outer membrane proteins" (POMP) since their precise location was not determined. The family is composed of two genes that are completely identical, and two genes with high homology to these genes. They calculated a molecular size of 90 and 91 kDa. The 5th encode a protein of 98 kDa. The sequence of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8) of C. pneumoniae were compared to the sequences of the C. Psittaci POMP proteins with the programme pileup in the GCG

package. The amino acid homologies were in the range of 51-63%. It is seen that the C. pneumoniae Omp4-5 proteins (SEQ ID NOs:2, 4) are most related to the 98 kDa POMP protein of C. psittaci. Interestingly, the 98 kDa C. psittaci POMP protein is more related to the C. pneumoniae genes than to the other C. psittaci genes. The repeated sequences of GGAI were conserved in the 98 kDa POMP protein, but only three GGAI repeats were present in the 90 and 91 kDa C. psittaci POMP proteins. For C.psittaci it has been shown that antibodies to these proteins seem to be protective for the infection.

Please replace the paragraph beginning at page 30, line 4 with the following amended paragraph:

The Omp4 (SEQ ID NO:1) gene was amplified by PCR with primers that contained LIC-sites, and the PCR product was cloned into the pET-30 LIC vector (Novagen). The histidine tagged fusion protein was expressed by induction of the synthesis by IPTG (isopropyl-beta-D-thiogalacto pyranoside) and purified over a nickel column. The purified Omp4 protein (SEQ ID NO:2) protein was used for immunization of a rabbit (six times, 8 µg each time).

Please replace the paragraph beginning at page 32, line 32 with the following amended paragraph:

Longbottom et al. (1996) have published partial sequence from 98 to 90 kDa proteins from *C. psittaci*. They have entered the full sequence of 5 genes in this family in the EMBL database. They have named the genes "putative outer membrane proteins" (POMP) since their precise location was not determined. The family is composed of two genes that are completely identical, and two genes with high homology to these genes. They calculated a molecular size of 90 and 91 kDa. The 5th encode a protein of 98 kDa. The sequence of the Omp4-7 proteins (SEQ ID NOs:2, 4, 6, 8) of *C. pneumoniae* were compared to the sequences of the *C. Psittaci* POMP proteins

with the programme pileup in the GCG package. The amino acid homologies were in the range of 51-63%. It is seen that the *C. pneumoniae* Omp4-5 proteins are most related to the 98 kDa POMP protein of *C. psittaci*. Interestingly, the 98 kDa *C. psittaci* POMP protein is more related to the *C. pneumoniae* genes than to the other *C. psittaci* genes. The repeated sequences of GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2) were conserved in the 98 kDa POMP protein, but only three GGAI (Gly-Gly-Ala-Ile, amino acids 164-167 of SEQ ID NO:2) repeats were present in the 90 and 91 kDa *C. psittaci* POMP proteins. For *C. psittaci* it has been shown that antibodies to these proteins seem to be protective for the infection.